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Heme oxygenase-1 attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts

Markus Wagner, Petra Cadetg, Rainer Ruf, Luca Mazzucchelli, Paolo Ferrari, and Claudio A. Redaelli

Department of Visceral and Transplantation Surgery, Department of Pathology, and Division of Nephrology and Hypertension, University of Bern, Bern, Switzerland

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Background. Kidneys can be preserved only for a limited time without jeopardizing graft function and survival. Induction of heat shock proteins (HSPs) can protect against ischemia/reperfusion (I/R) injury. Therefore, we investigated whether the induction of the HSP, heme oxygenase-1 (HO-1), improves outcome following isotransplantation after an extended period of cold storage.

Methods. Rats were subjected to heat preconditioning (HP; 42°C for 20 minutes). Kidneys harvested after 24 hours, were preserved in cold University of Wisconsin (UW) solution at 4° C for 45 hours and transplanted into bilateral nephrectomized rats. Cobalt protoporphyrin (CoPP) was administered in another group of animals in order to induce HO-1 pharmacologically, while other groups of animals received the HO-1 inhibitor, tin protophorphyrine (SnPP), following HP or CoPP.

Results. Cold ischemia caused a complete attenuation of graft function within 3 days following transplantation and subsequent death of all animals, whereas HP protected graft function and five of nine rats survived for 3 weeks. HP inhibited the induction of osteopontin and induced the expression of HO-1, HSP 70 and 90, and the antiapoptotic factor Bcl-X_L. Grafts exposed to HP were protected against structural I/R injuries as revealed by histologic assessment using a semiquantitative score. Furthermore, induction of apoptosis was attenuated and activation of caspase-3 was inhibited. Comparable results were observed after administration of CoPP, whereas SnPP inhibited the effects of HP and CoPP.

Conclusion. HP or administration of CoPP induced both HO-1, preserved kidney graft function, and prevented postreperfusion apoptosis after cold preservation.

End-stage kidney disease (ESRD) can be treated successfully by renal transplantation, but the number of patients eligible for this treatment far exceeds the number of available organs. Therefore, efforts to improve organ preservation and improve graft function are important tools in order to ameliorate organ shortage. Extended cold preservation typically causes proximal tubular and medullary damage in the kidney with cytoskeletal alterations, mitochondrial injury, and consecutive impaired graft function [1–2]. Introduction of the University of Wisconsin (UW) solution more than 10 years ago considerably extended the time of cold storage between harvesting and transplantation [3–5]. However, preservation of organs, especially of borderline kidneys, for more than 24 hours remains a risk factor for primary graft dysfunction and even nonfunction [6, 7].

One of the more promising strategies to increase cold ischemia tolerance is exposure to transient sublethal hyperthermia referred to as heat preconditioning (HP). There is increasing evidence that HP provides cytoprotective effects on ischemia/reperfusion (I/R)–induced injury in various systems by induction of a family of so-called heat shock proteins (HSPs) [8]. HSPs are ubiquitous and comprise several structurally unrelated groups of proteins named according to their apparent molecular weight, such as HSP 32, HSP 70 or HSP 90 [9–11]. However, induction of HSP expression is caused by almost any kind of stress and seems to protect the function and structural integrity of the cells [12–17]. HSP 70 is among the best characterized member of the HSP family. These proteins include constitutively expressed isoforms, such as HSC 70, and stress-induced isoforms, such as HSP 72 [18]. In addition, heme oxygenase (HO), a crucial enzyme of heme metabolism, represents another HSP protein with a size of 32 kD. So far, three isoforms have been identified, named HO-1, HO-2, and HO-3 [19–21]. Of these, HO-1 catalyzes the breakdown of heme into equimolar amounts of biliverdin, carbon monoxide, and iron [22, 23]. Bilirubin, the metabolite of biliverdin, is a potent endogenous antioxidant and carbon monoxide functions as a signaling molecule [22]. Many protective effects of HSPs have been attributed to overexpression

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of HO-1, whose expression can be induced by pharmacologic manipulations. For example, administration of a spin trap agent induced HO-1 and attenuated oxidative injury in a rat kidney I/R model [24]. Furthermore, induction of HO-1 by administration of cobald protoporphyrin (CoPP) protected transplanted fatty Zucker rat livers from I/R injury [25]. In addition, up-regulation of HO-1 by CoPP suppressed immune effector functions such as T-cell–mediated and natural killer (NK)-cell–mediated cytotoxicity and inhibited differentiation of cytotoxic T cells [26].

Recently, apoptosis has been identified as a major component of reperfusion injuries [27, 28]. The induction of apoptosis involves a cascade of several proapoptotic factors like caspases and antiapoptotic factors like Bcl-X_L. HSPs can interfere with several steps of the apoptosis pathway, suggesting that their induction could prevent reperfusion-induced apoptosis [29, 30]. Therefore, we investigated whether the induction of HO-1 improves the outcome of renal transplantation using an experimental, syngeneic rat model. We analyzed whether HP preserves graft function after prolonged cold ischemia. To evaluate a clinically feasible approach, we assessed whether induction of HO-1 by pharmacologic agents such as CoPP may reproduce the effects of HP. In addition, we examined whether induction of HO-1 affected reperfusion-induced apoptosis.

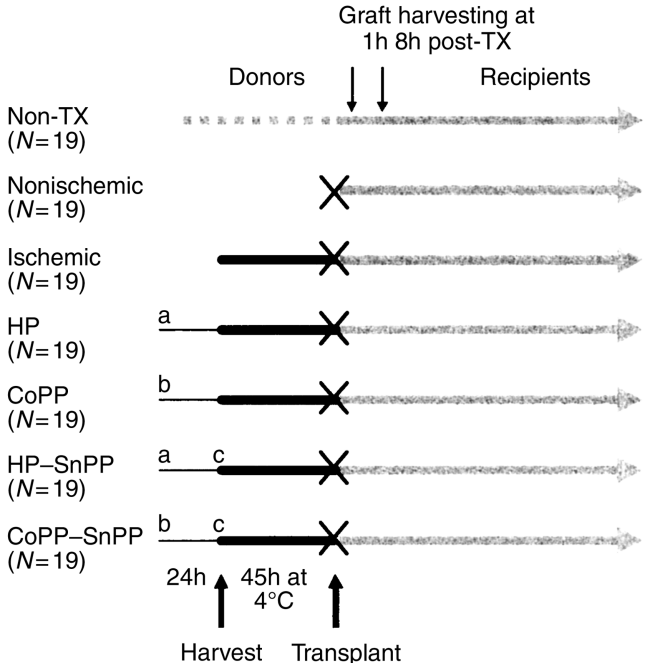

Fig. 1. Experimental flow chart. Experimental flow chart for the orthotopic kidney transplantation isogeneic model (Lewis–Lewis) with interventions in the donor and sampling in the recipients. Each group consisted of 19 animals, five rats were sacrificed at 1 hour and 8 hours after transplantation, respectively, for the analysis of gene expression, histologic changes, and apoptosis in the graft. Survival and renal function were assessed in the remaining animals in each group. Donor were pretreated as follows: Heat preconditioning (HP) (a) for 20 minutes at 42°C 24 hours before harvesting; intraperitoneal cobalt-protoporphyrin (CoPP) (b) 24 hours before harvesting; tin-Protoporphyrin (SnPP) (c) 23 hours after HP or CoPP. Abbreviations are: Non-TX, nontransplanted controls; nonischemic-TX, transplanted rats without cold ischemia; ischemic-TX, transplanted rats with cold ischemia; HP, transplanted rats with cold ischemia after heat preconditioning (HP); CoPP, transplanted rats with cold ischemia after cobalt protoporphyrin (CoPP); HP-SnPP, transplanted rats with cold ischemia after HP and treatment with tin protophorphyrine (SnPP); CoPP-SnPP, transplanted rats with cold ischemia after CoPP and treatment with SnPP.

METHODS

Animals and treatments

Male Lewis rats (Harlan, Horst, The Netherlands) weighing 250 to 300 g were allowed free access to regular chow and water. Animal experiments were performed in accordance with the regulations for the care and use of laboratory animals and were approved by the local authorities. Seven groups of rats were studied. The control groups consisted of a nonstressed, nontransplanted control group (non-TX) ($N = 19$), renal transplanted rats without graft exposure to cold ischemia (nonischemic-TX) ($N = 19$) and renal transplanted animals with 45 hours cold ischemia of the graft (ischemic-TX) ($N = 19$). The experimental groups all received a renal isograft after 45 hours cold ischemia and were pretreated as follows: HP ($N = 19$), or pretreatment with CoPP ($N = 19$) alone or together with the HO-1 inhibitor tin protoporphyrin (SnPP) after HP (HP-SnPP) ($N = 19$) or after CoPP (CoPP-SnPP ($N = 19$) (Fig. 1).

For HP, donor animals but not recipients, were exposed to hyperthermia using a heat pad and infrared lamp after induction of general anesthesia by intraperitoneal injection of pentobarbitone sodium (50 mg/kg body weight). Body temperature was monitored with a rectal thermometer and temperature was slowly increased to 42°C and maintained for 20 minutes. After HP and sham HP, all animals were given 5 mL isotonic saline intraperitoneally to avoid dehydration. Harvesting of the kidneys was performed 24 hours following hyperthermia exposure. For pharmacologic induction of HO-1, donor animals received 5 mg/kg of CoPP intraperitoneally 24 hours before organ harvesting. To inhibit HO-1 induction without toxicity, 10 μmol/kg SnPP (Porphyrin Products, Logan, UT, USA), solubilized in 0.1 mol/L NaOH and diluted 1:1 in phosphate-buffered saline (PBS) (pH 7.4), were administered intraperitoneally 23 hours following HP or CoPP [31]. Animals from each group were sacrificed at 1 hour ($N = 5$ per group) and 8 hours ($N = 5$ per group) after transplantation to study protein expression, histologic changes, and apoptosis. Survival and graft function, which were observed until death or 3 weeks following transplantation, were assessed in the remaining nine animals of each group. Urine volume, urine protein content, and urine creatinine levels were determined daily. Jugular venous blood was collected every third day

in order to determine serum creatinine concentration, urea and protein levels, and to estimate glomerular filtration rate (GFR) by the creatinine clearance method.

Surgical procedures

For organ harvesting, a midline laparotomy was performed and the infradiaphragmatic aorta was clamped. Kidneys were gently prepared, then flushed with chilled lactated Ringer's solution, and thereafter perfused for 5 minutes with 20 mL 4°C cold University of Wisconsin (UW) solution via the renal artery and removed. Kidneys were cooled with UW solution and stored at 4°C for 45 hours. For kidney transplantation, recipients were anesthetized with pentobarbitone sodium (50 mg/kg body weight), both recipient kidneys were removed and single isograft kidney transplantation was performed. Renal vessels were sutured using 10/0 Prolene (Ethicon, Somerville, NJ, USA) and ureters were connected via intraluminal polyethylene cuffs. Transplantation time ranged between 15 and 20 minutes in all animals. To eliminate operator-related discrepancy, all transplant procedures were performed by a single investigator (J-H.T.). After transplantation, all animals were kept in metabolic units for renal function analysis.

Western blot analysis for renal osteopontin, HSP 90, HSP 70, HO-1 and Bcl-X_L expression

For osteopontin immunoblotting, kidney tissue was homogenized in 25 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, and 10 mmol/L ethylenediaminetetraacetic acid (EDTA) containing protease inhibitors (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Aliquots containing 40 μg protein were subjected to sodium dodecyl sulfate (SDS)/12% polyacrylmide gel electrophoresis (PAGE), transferred to nitrocellulose membranes and blotted with a monoclonal antiosteopontin antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) diluted 1:1000, washed and subjected to chemiluminescence Western blotting using a commercially available blotting kit (Pierce Chemical Co., Rockford, IL, USA) and a horseradish peroxidase-conjugated goat antirabbit antibody (Developmental Studies Hybridoma Bank). The intensity of the Western blot signal was quantified with a charged coupling device (CCD) camera (Fujifilm LAS-1000, Raytest, Urdorf, Switzerland) using the software AIDA 2.1 (Raytest) and normalized to β-tubulin (H235) protein expression levels (rabbit polyclonal IgG, 200 μg/mL; Santa Cruz Biotechnology, Heidelberg, Germany) in order to equalize differences in protein loading.

For determination of HSP 90, HSP 70, HO-1 and Bcl-X_L protein expression, the kidney was minced and suspended in four volumes of 0.25 mol/L sucrose at 4°C and homogenized two times for 30 seconds with a Polytron (Janke & Kunken KG IGA Werk, Staufen, Germany). Homogenates were subjected to SDS/5% PAGE, transferred to nitrocellulose membranes and probed with polyclonal antibodies (HSP 70 1:1000 and HSP 90 1:1000, Stressgen, Victoria, British Columbia, Canada; and Bcl-X_L 1:1000, Transduction Laboratories, Lexington, KY, USA). Membranes were washed and subjected to chemiluminescence Western blotting and quantification as described above (donkey-antirabbit secondary antibody; Pierce, Lausanne, Switzerland).

Histopathologic analysis

To determine the histologic degree of ischemic kidney injury, specimens from grafts 1 and 8 hours after reperfusion were fixed in buffered formalin (4%), embedded in paraffin, and 4 μm thick tissue sections were cut and stained with hematoxylin and eosin. Tubulointerstitial tissue damage was evaluated using a semiquantitative scoring system, as described previously in detail [24]. Seven different histologic parameters, such as cell swelling, nuclear pyknosis, cell lysis, cell sloughing, loss of proximal tubules brush border, as well as the presence of protein casts and neutrophilic tubulitis. were analyzed.

In situ detection of apoptosis using the TUNEL assay

Apoptosis was determined and quantified by terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling (TUNEL) assays. TUNEL staining was performed 1 and 8 hours after reperfusion. Baseline TUNEL staining were performed in contralateral donor kidneys. Samples embedded in paraffin were cut to 4 μm sections and deparaffinized after affixing to glass slides by soaking twice in xylol 100% for 10 minutes. Thereafter, the sections were treated for 30 minutes with 4% diethylpyrocarbonate in ethanol at 4°C in order to avoid false positivity, as described elsewhere [32]. The samples were successively soaked in ethanol 100% (2 × 10 minutes), ethanol 94% (5 minutes), ethanol 70% (5 minutes), ethanol (50%), and double-distilled water (5 minutes). After treatment with proteinase K (10 μg/mL) at 37°C for 60 minutes in 5 mmol/L EDTA in Tris 20 mmol/L (pH 8.1), the sections were incubated for 30 minutes in methanol containing H_2O_2 0.3% to inhibit endogenous peroxidase activity. TUNEL assays were performed using a commercial kit (Roche Diagnostic, Basel, Switzerland). The sections were treated with terminal nucleotidyl transferase in the presence of fluorescein-deoxynucleotidyl transferase-mediated triphosphate (dUTP) and desoxynucleoside triphosphate (dNTP) and incubated with horseradish peroxidase-conjugated antifluorescein antibody (Roche, Basel) and developed using 3-amino-9-ethylcarbazol in solution ready to use with substrate chromogene (Envision System Kit, DAKO). Tissues treated with DNAse I served as positive controls and sections stained without terminal nucleotidyl transferase as negative controls. The specimen were counter-

Table 1. Survival in control and experimental animals ($N = 9$)

Treatment groups	Mean survival *days*	Mortality (N) at the end of study	P value
Control			
Non-TX (non-stressed, non-transplanted)	21	0	
Non-ischemic TX	21	0	
Ischemic TX	2	9 (100%)	
Experimental groups			
HP (heat preconditioning)	14	5 (55%)	<0.01[a]
CoPP (Cobalt protoporphyrin)	13	4 (44%)	<0.01[a]
HP-SnPP (HP + Tin-protoporphyrin)	4	9 (100%)	<0.001[b]
CoPP-SnPP (CoPP + Tin-protoporphyrin)	3	9 (100%)	<0.001[c]

[a]Survival in animals exposed to HP or CoPP compared to control (ischemic TX)
[b]Survival after administration of HP-SnPP in comparison to transplanted rats with cold ischemia after HP
[c]Survival in comparison to transplanted rats with cold ischemia after administration of CoPP

stained with hematoxylin and mounted with Aquamount. The degree of apoptosis was estimated by point counting. TUNEL-positive tubular cells were counted in 10 randomly selected microscopic fields (×400) per sample and expressed as percentages of the total number of tubular cells.

Immunohistochemistry for activated caspase 3

Paraffin-embedded sections were cut, deparaffinized, and hydrated by soaking in xylol 100% (2 × 10 minutes), ethanol 100% (2 × 10 minutes), ethanol 94% (5 minutes), ethanol 70% (5 minutes), ethanol (50%), and double-distilled water (5 minutes). To prevent staining by endogenous peroxidases, sections were incubated for 30 minutes in methanol containing H_2O_2 0.6% and washed for 3 minutes in double-distilled water and twice for 5 minutes in TBS (pH 7.4) containing 0.1% bovine serum albumin (BSA). Sections were blocked for 30 minutes with normal goat serum (Kirkegard & Perry, Gaithersburg, MD, USA) and incubated overnight at 4°C with the primary antibody, a rabbit polyclonal antibody recognizing specifically the activated caspase 3 (1:2000 CM1 antibody, IDUN Pharmaceuticals, Inc., La Jolla, CA, USA). After washing with TBS containing BSA, the sections were incubated for 30 minutes at room temperature with the secondary antibody, peroxidase-labeled polymer conjugated to goat antirabbit immunoglobulins (DAKO kit K4008) and exposed for 15 minutes to 3-amino-9-ethylcarbazol in solution ready to use with substrate chromogene (Envision System kit, DAKO). Sections from mammary gland in involution served as positive control and the first antibody was omitted in negative controls. The sections were counterstained with hematoxylin and mounted with Aquamount. The activation of the caspase 3 was assessed by point counting (2000 points per graft).

Statistics

Data are expressed as mean ± SEM. Graft survival was calculated with the Kaplan-Meier product limit estimator. Differences in survival rates between the groups were tested with the log rank test in a statistical package program (SPSS Statistical Software, Chicago, IL, USA). Differences between two groups were identified by unpaired two-tailed Student t test. Comparisons of three or more experimental groups were performed by analysis of variance using a Fisher test. A P value < 0.05 was considered statistically significant.

Table 2. Reasons for non-survival among heat preconditioned (HP) and Cobalt-protoporphyrin pretreated (CoPP) rats

Mortality	Heat precondition ($N = 9$)	Cobalt protoporphyrin ($N = 9$)
Overall mortality	4 (44%)	5 (55%)
Uremia	2	1
Bleeding	1	1
Sepsis/infection	1	2
Unknown		1

RESULTS

Animal survival

Mean survival was calculated from nine animals in each group (Table 1). Isografting without exposition to cold ischemia did not lead to recipient death (nonischemic-TX). In contrast, exposition to cold ischemia was inevitably associated with death of the recipient animals within 3 days following transplantation (ischemic-TX). HP or administration of CoPP both increased survival significantly in comparison to control recipients receiving kidney grafts after exposure to 45 hours of cold ischemia (ischemic-TX) (Table 1). In contrast, the effects of HP as well as administration of CoPP on survival were attenuated by administration of SnPP (Table 1). Four animals in the HP-TX group and five animals in the CoPP group did not survive up to 21 days. The reasons for death are outlined in Table 2. Death for reasons other than uremia but graft-nonfunction was observed in two rats of the HP group and four rats of the CoPP group.

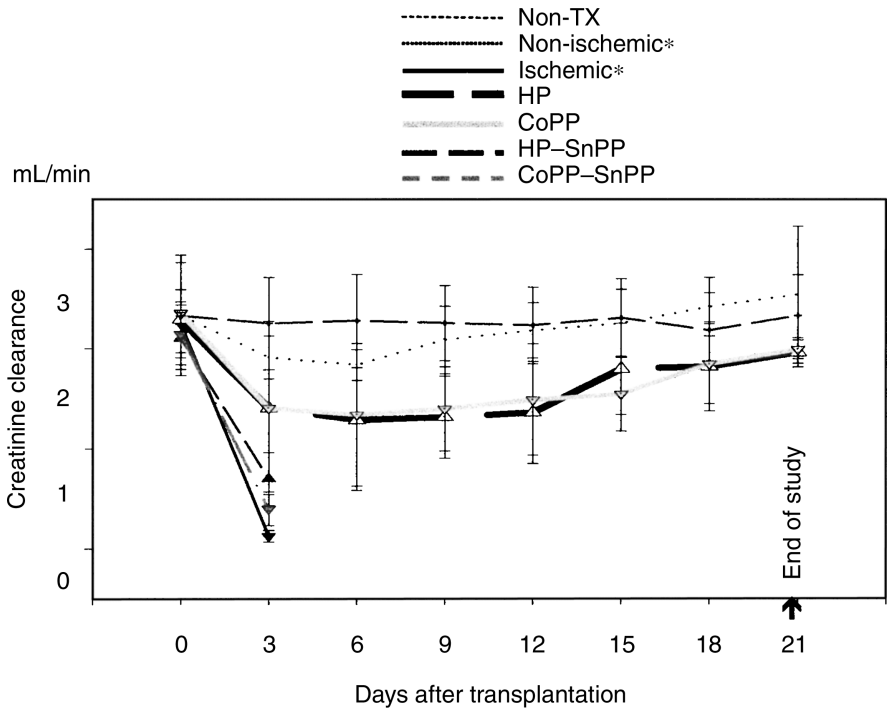

Fig. 2. Creatinine clearance following kidney isotransplantation. Mean ± SEM are from values of animals surviving within each group until the end of the observation period. Abbreviations are: Non-TX, nontransplanted controls; nonischemic-TX, transplanted rats without cold ischemia; ischemic-TX, transplanted rats with cold ischemia; HP, transplanted rats with cold ischemia after heat preconditioning (HP); CoPP, transplanted rats with cold ischemia after cobalt protoporphyrin (CoPP); HP-SnPP, transplanted rats with cold ischemia after HP and treatment with tin protophorphyrine (SnPP); CoPP-SnPP, transplanted rats with cold ischemia after CoPP and treatment with SnPP.

Kidney graft function

For all groups, renal function data at day 0 shown in Figure 2 are mean GFR data before transplantation. GFR of nontransplanted animals averaged 2.26 ± 0.41 mL/min during the whole experimental period. GFR of nonischemic-TX rats was slightly lower on day 3 (1.91 ± 0.21 mL/min) and day 6 (1.84 ± 0.15 mL/min) and thereafter increased to the same levels until the end of study (2.54 ± 0.69 mL/min). Cold ischemia caused a total loss of GFR within 3 days following transplantation in all recipients in the ischemic-TX group, whereas HP preserved creatinine clearance in HP-TX rats, reaching minimal values on day 6 following transplantation (1.29 ± 0.71 mL/min). Thereafter, values increased until the end of the study period but remained significantly lower in comparison to nonischemic-TX animals (day 21, 1.9 ± 0.71 mL/min, $P < 0.05$). Similar results were observed after administration of CoPP with minimal GFR values of 1.32 ± 0.52 mL/min at day 6. Administration of SnPP attenuated the effects of either HP-SnPP or CoPP-SnPP with a marked decrease of GFR at day 3 (HP-SnPP, 0.7 ± 0.71 mL/min; CoPP-SnPP, 0.4 ± 0.71 mL/min).

Immunoblotting analysis

Intragraft osteopontin (OPN) protein levels (Table 3) of nonischemic-TX rats did not alter during the first 8 hours following organ reperfusion and were comparable to baseline expression in non-TX animals. In contrast, cold ischemia caused a massive up-regulation of OPN protein expression levels within 8 hours following reperfusion in the ischemic-TX group. Both HP and CoPP inhibited induction of OPN expression markedly in comparison to ischemic-TX (HP, $P < 0.0001$; and CoPP, $P < 0.0001$). Administration of SnPP reversed the effect of HP and CoPP (HP-SnPP and CoPP-SnPP, respectively). The expression of HSP 90 and HSP 70 and HO-1 was markedly increased 1 hour and 8 hours after transplantation in grafts subjected to HP compared to ischemic-TX (Fig. 3). In contrast, graft preconditioning with CoPP caused selectively the induction of HO-1 (Fig. 3). Bcl-X_L protein expression levels were not altered 1 hour and 8 hours following isografting in nonischemic-TX animals (Table 4). Similar, Bcl-X_L expression was unaltered after 1 hour and 8 hours following isografting in ischemic-TX (Table 4). In contrast, HP and CoPP both caused a tremendous increase of Bcl-X_L expression levels (Table 4). Administration of SnPP to either HP or CoPP completely attenuated this induction of Bcl-X_L (Table 4).

Histology

Nonischemic isografting caused minimal changes in kidney histology compared to non-TX controls as assessed by using a semiquantitative scoring system (Fig. 4; nonischemic-TX vs. non-TX). In contrast, ischemic-TX led to severe histologic alterations 1 hour and 8 hours following organ reperfusion, while either HP or CoPP prevented ischemia-induced structural damage (Fig. 4; HP and CoPP). Administration of SnPP abolished the protective effect of HP and CoPP (Fig. 4; HP-SnPP and CoPP-SnPP), although the observed changes were not as extensive as seen in ischemic-TX rats.

Table 3. Relative levels of intragraft osteopontin protein expression following rat kidney graft transplantation at 1 hour and 8 hour after transplantation. Time 0 are values from the corresponding contralateral donor kidney (osteopontin expression values are normalized to β-tubulin and are given as mean ± SE; $N = 5$)

Treatment group	0 hour	1 hour	8 hour
Controls			
Non-TX	0.22 ± 0.02	0.32 ± 0.03	0.53 ± 0.02
Non-ischemic TX	0.37 ± 0.02	0.45 ± 0.02	0.55 ± 0.06
Ischemic TX	0.36 ± 0.03	0.51 ± 0.05	4.7 ± 0.9[a]
Experimental groups			
HP (heat preconditioning)	0.45 ± 0.02	0.50 ± 0.04	1.2 ± 0.25[b]
CoPP (Cobalt protoporphyrin)	0.50 ± 0.03	0.34 ± 0.03	1.4 ± 0.21[b]
HP-SnPP (HP + Tin-protoporphyrin)	0.45 ± 0.03	0.41 ± 0.04	2.5 ± 0.29[c]
CoPP-SnPP	0.58 ± 0.03	0.29 ± 0.03	4.3 ± 0.87[c]

[a] In comparison to non-ischemic TX ($P < 0.05$)
[b] Animals exposed to HP or CoPP compared to controls (ischemic TX, $P < 0.05$)
[c] Animals exposed to HP + SnPP or CoPP + SnPP in comparison to HP and CoPP, respectively ($P < 0.05$)

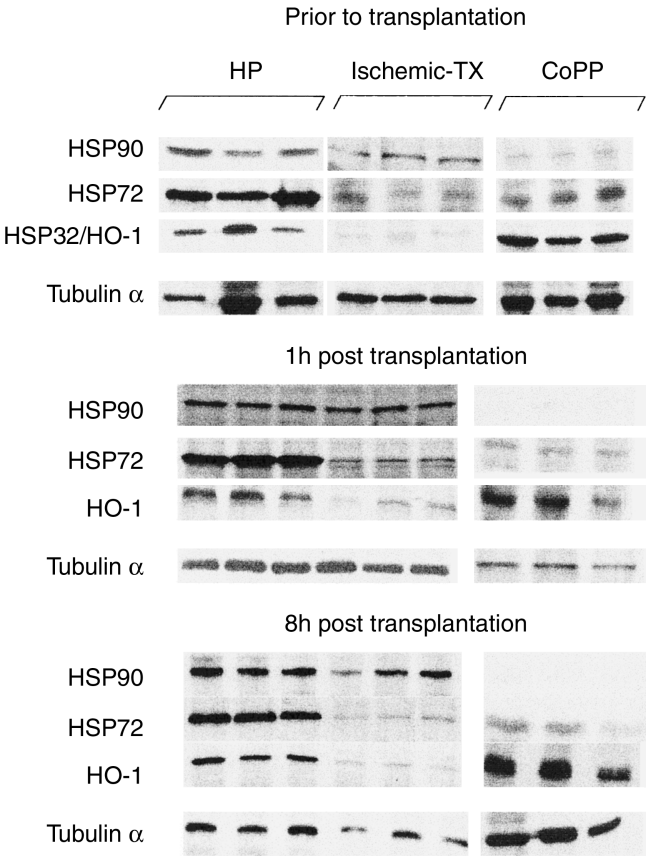

Fig. 3. Heat shock protein (HSP) immunoblotting studies. Immunoblot analysis of HSP 70, HPS 90, and HSP 32/heme ocygenase-1(HO-1) expression following kidney isotransplantation after prolonged cold ischemia. Blots are known for heat preconditioned (HP), Cobalt protoporphyrin preconditioned (CoPP) animals and for ischemic control rats. The amount of protein loaded was adjusted for immunoreactive tubulin α.

Table 4. Relative levels of intragraft Bcl-X_L expression following rat kidney graft transplantation (Bcl-X_L expression values are normalized to β-tubulin and are given as mean ± SE; $N = 5$)

Treatment group	0 hour	1 hour	8 hour
Controls			
Non-TX	0.06 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Non-ischemic TX	0.27 ± 0.03	0.36 ± 0.02	0.22 ± 0.02
Ischemic TX	0.32 ± 0.02	0.16 ± 0.01	0.21 ± 0.02
Experimental groups			
HP (heat preconditioning)	0.48 ± 0.02	1.6 ± 0.25[b]	2.02 ± 0.34[b]
CoPP (Cobalt protoporphyrin)	0.43 ± 0.03	1.56 ± 0.23[b]	2.48 ± 0.28[b]
HP-SnPP (HP + Tin-protoporphyrin)	0.22 ± 0.01	0.41 ± 0.04	0.35 ± 0.07
CoPP-SnPP	0.23 ± 0.01	0.29 ± 0.03	0.38 ± 0.15

[a] In comparison to non-ischemic TX ($P < 0.05$)
[b] Animals exposed to HP or CoPP compared to controls (ischemic TX, $P < 0.05$)

Assessment of apoptosis (TUNEL assay) and determination of activated caspase 3 protein expression

TUNEL-positive tubular epithelial cells were rarely observed in nonischemic isografts (Fig. 5). In contrast, ischemia caused a rapid increase of apoptotic cells in ischemic-TX rats (Fig. 5). Both HP or CoPP significantly reduced the induction of apoptosis at 1 hour and 8 hours after kidney transplantation (Fig. 5; HP and CoPP). This effect was attenuated by SnPP administration (Fig. 5; HP-SnPP and CoPP-SnPP). Caspase 3 was expressed at low levels in nonischemic-TX controls (Fig. 6). However, 45 hours of cold storage caused a strong induction of caspase 3 activity (Fig. 6; ischemic-TX) which was attenuated by either HP or administration of CoPP (Fig. 6; HP and CoPP). Administration of SnPP to the donor animal abolished these effects (Fig. 6; HP-SnPP and CoPP-SnPP).

DISCUSSION

The present findings in a model of syngeneic renal transplantation in rats suggest (*1*) that HP permits longer cold storage of the harvested kidney without compromising survival, (*2*) that pharmacologic induction of HO-1 and other HSPs by CoPP achieves the same beneficial effect as HP, and (*3*) that preconditioning with either HP or CoPP prevents reperfusion-induced microstructural changes and apoptosis in renal grafts.

Currently, storage of kidney grafts in UW solution beyond 24 hours is envisioned with decreased organ function and even nonfunction. We found that prolonged cold ischemia of 45 hours without preconditioning inevitably caused graft failure and death of the recipient animals. On the other hand, cold ischemia following HP or administration of CoPP was well tolerated and had little influence on graft function. Thus, in surviving animals, GFR decreased transiently but was comparable to non-

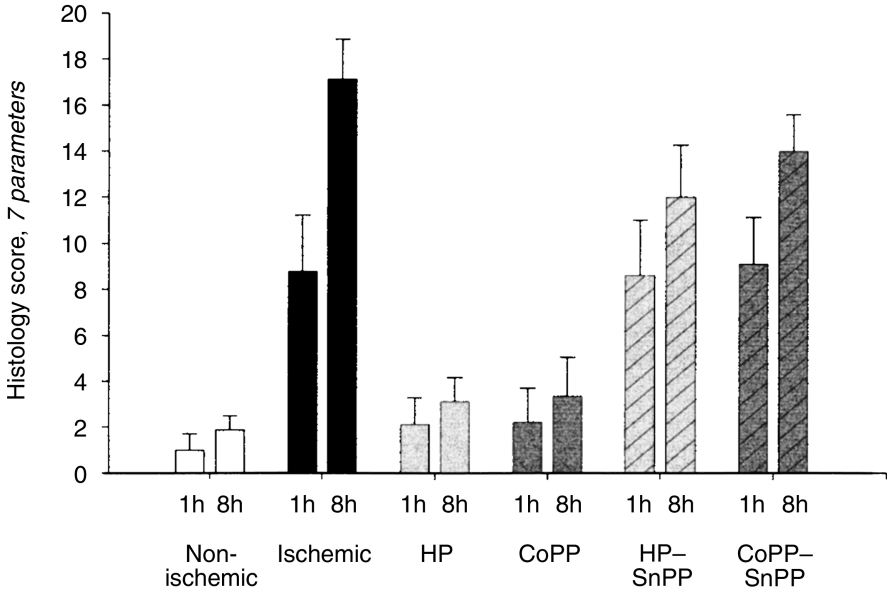

Fig. 4. Histologic assessement of ischemic graft injury. To determine the histologic degree of ischemic kidney injury, specimens from grafts 1 hour and 8 hours after reperfusion were assessed for tissue damage using a semiquantitative scoring system. Seven different histologic parameters such as cell swelling, nuclear pyknosis, cell lysis, cell sloughing, loss of proximal tubules brush border, as well as the presence of protein casts and neutrophilic tubulitis were analyzed. Abbreviations are: nonischemic-TX, transplanted rats without cold ischemia; ischemic-TX, transplanted rats with cold ischemia; HP, transplanted rats with cold ischemia after heat preconditioning (HP); CoPP, transplanted rats with cold ischemia after cobalt protoporphyrin (CoPP); HP-SnPP, transplanted rats with cold ischemia after HP and treatment with tin protophorphyrine (SnPP); CoPP-SnPP, transplanted rats with cold ischemia after CoPP and treatment with SnPP.

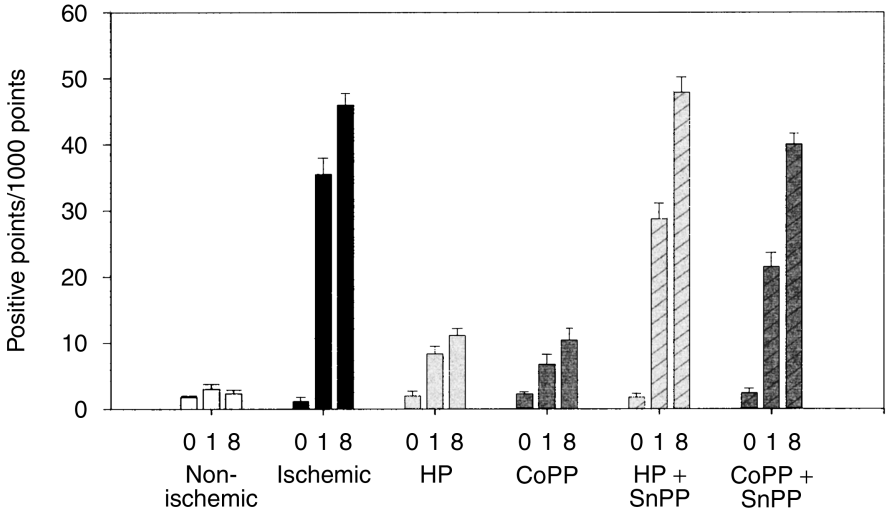

Fig. 5. Terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling (TUNEL) assay in kidney grafts. Determination of apoptosis in kidney isografts by TUNEL assay 1 hour and 8 hours following transplantation. Time 0 are values from the corresponding contralateral donor kidney. Abbreviations are: nonischemic-TX, transplanted rats without cold ischemia; ischemic-TX, transplanted rats with cold ischemia; HP, transplanted rats with cold ischemia after heat preconditioning (HP); CoPP, transplanted rats with cold ischemia after cobalt protoporphyrin (CoPP); HP-SnPP, transplanted rats with cold ischemia after HP and treatment with tin protophorphyrine (SnPP); CoPP-SnPP, transplanted rats with cold ischemia after CoPP and treatment with SnPP.

ischemic transplanted rats at day 21 following grafting. Furthermore, preconditioning by HP or CoPP was associated with decreased expression of osteopontin and inhibition of I/R damages and apoptosis as revealed by histologic assessment and immunohistochemistry. However, not all animals receiving allografts treated with either HP or CoPP followed by 45 hours cold ischemia survived. Uremia was the cause of death in only a minority of animals while surgical or infectious complications accounted for two thirds of failures in these rats (Table 1). Therefore, one may hypothesize that induction of HO-1 by HP or CoPP may preserve graft function and survival more strongly than actually observed in the present study. In fact, function of grafts treated by HP or CoPP in surviving animals was comparable to transplanted rats whose isografts were not exposed to cold ischemia. At present it remains unclear why surgical and infectious complications were seen in the HP- and CoPP-pretreated groups but not in the nonischemic controls. There is no actual scientific evidence that induction of HO-1 may increase the susceptibility to infections. In contrast, there is increasing evidence that HO-1 induction may protect against endotoxin-induced tissue damage and decrease mortality in experimental endotoxemia [33–37]. For instance, induction of TNF-α plays an important role in endotoxin-related mortality and HO-1 is protective against TNF-α–induced cytotoxicity [38–40]. Therefore, it seems unlikely that heat shock–induced TNF-α production may be responsible for the observed mortality.

The present findings in a rat renal transplantation

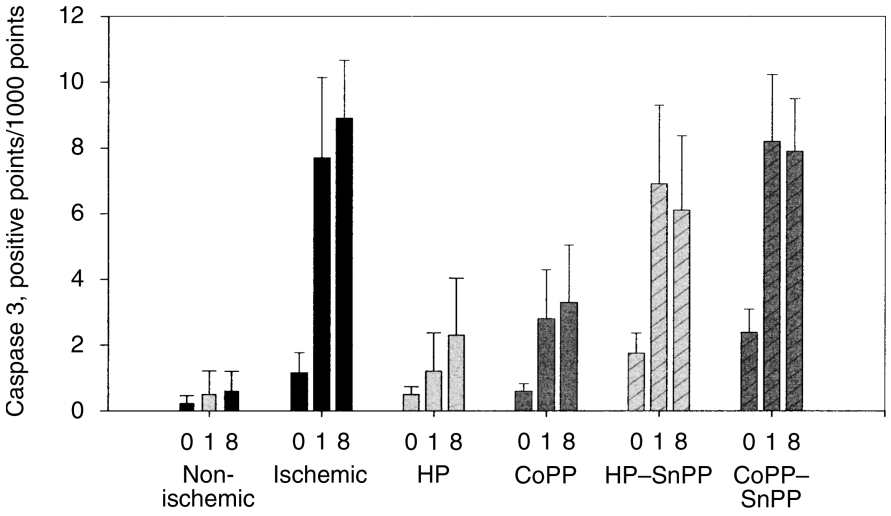

Fig. 6. Activated caspase 3 immunohistochemistry. Activated caspase-3 was assessed 1 hour and 8 hours after transplantation. Time 0 are values from the corresponding contralateral donor kidney. Abbreviations are: Non-TX, nontransplanted controls; nonischemic-TX, transplanted rats without cold ischemia; ischemic-TX, transplanted rats with cold ischemia; HP, transplanted rats with cold ischemia after heat preconditioning (HP); CoPP, transplanted rats with cold ischemia after cobalt protoporphyrin (CoPP); HP-SnPP, transplanted rats with cold ischemia after HP and treatment with tin protophorphyrine (SnPP); CoPP-SnPP, transplanted rats with cold ischemia after CoPP and treatment with SnPP.

model confirm the results of previous studies, which analyzed the effects of HO-1 in experimental liver transplantation [41, 42]. Our results seem to reveal a central role of HO-1 as a protective agent for transplanted kidney grafts. Moreover, comparable results from HP and CoPP pretreatment demonstrate that pharmacologic induction of HO-1 might be of clinical value to permit longer cold storage of harvested organs prior to transplantation. While heat preconditioning may not be achievable in a clinical setting, pretreatment with CoPP could be an interesting alternative. Further experimental studies are mandatory to demonstrate the safety and efficacy of CoPP preconditioning before organ retrieval. Several mechanisms of action might explain the protective effect of HO-1 and other HSPs. For example, HO-1 has been suggested to exert inducible functions as a cellular protector against oxidative stress [43, 44]. Its three metabolites, bilirubin, free iron, and carbon monoxide, are all active compounds. For example, bilirubin has antioxidant properties and carbon monoxide stimulates the formation of cyclic guanosine monophosphate (cGMP) [45, 46]. This second messenger protect kidney grafts from renal platelet accumulation after I/R [47]. HO-1 also exerts immunosuppressive properties, which are relevant for transplantation. Its induction inhibited lymphoproliferative alloresponse and differentiation of cytotoxic T cells and prolonged heterotopic heart allograft survival [26]. Furthermore, Soares et al [48] reported that expression of HO-1 enhanced long-term survival in a model of cardiac xenotransplantation.

Apoptosis was found to be a key feature of I/R injury following transplantation [49–53]. Several HSPs interact directly with various components of the apoptotic pathway. For instance, hepatic sinusoidal endothelial cells are particularly vulnerable to apoptosis [27, 28] and damage to endothelial cells may lead to secondary injury and apoptosis of the hepatocytes and depending on the extension of this process may impair graft function [54]. Carbon monoxide, a product of HO-1, has been shown to suppress endothelial cell apoptosis in vitro by activation of p38 mitogen-activated protein kinase [55]. Furthermore, HO-1 transgenic mice overexpressed the antiapoptotic Bcl-2 [56]. In addition, HO-1 gene transfer was reported to attenuate lung injury via a caspase-mediated pathway [57, 58]. Due to the known antiapoptotic properties of HO-1, we investigated whether induction of HO-1 by HP or CoPP could prevent apoptosis in grafts after extended cold ischemia. There was a marked reduction in apoptotic cells in grafts retrieved 1 hour and 8 hours after reperfusion in grafts that underwent HP exposure or CoPP administration. In correlation with the results from the TUNEL assays, there were markedly fewer cells positive for activated caspase 3 and an increase in the expression of the antiapoptotic protein Bcl-X_L 1 hour and 8 hours after reperfusion. These data, as well as other recent reports, suggest that prevention of cell apoptosis may offer a therapeutic benefit in improving the outcome of renal transplants [59, 60].

CONCLUSION

The present findings indicate a beneficial role for HO-1 and other HSP in an experimental rat kidney transplantation model by enhancing the tolerance of prolonged cold ischemia. Pharmacologic induction of HO-1 prior to organ harvesting is an attractive approach to preserve graft function and increase survival in the transplantation setting, in particular, by preventing apoptosis. Therefore, modulation of HO-1 expression promises clinical potential and may have a role in order to ameliorate the increasing shortage of suitable organs for patients with end-stage kidney disease.

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Reprint requests to Claudio A. Redaelli, M.D., Department for Visceral and Transplantation Surgery, University of Bern, Bern, Switzerland. E-mail: claudio.redaelli@insel.ch

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